Engineering of an FGF–proteoglycan fusion protein with heparin-independent, mitogenic activity

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In the absence of heparan sulfate (HS) on the surface of target cells, or free heparin (HP) in the vicinity of their receptors, fibroblast growth factor (FGF) family members cannot exert their biological activity and are easily damaged by proteolysis. This limits the utility of FGFs in a variety of applications including treatment of surgical, burn, and periodontal tissue wounds, gastric ulcers, segmental bony defects, ligament and spinal cord injury. Here we describe an FGF analog engineered to overcome this limitation by fusing FGF-1 with HS proteoglycan (PG) core protein. The fusion protein (PG–FGF-1), which was expressed in Chinese hamster ovary cells and collected from the conditioned medium, possessed both HS and chondroitin sulfate sugar chains. After fractionation, intact PG–FGF-1 proteins with little affinity to immobilized HP and high-level HS modification, but not their heparitinase or heparinase digests, exerted mitogenic activity independent of exogenous HP toward HS-free Ba/F3 transfectants expressing FGF receptor. Although PG–FGF-1 was resistant to tryptic digestion, its physiological degradation with a combination of heparitinase and trypsin augmented its mitogenic activity toward human endothelial cells. The same treatment abolished the activity of simple FGF-1 protein. By constructing a biologically active proteoglycan–FGF-1 fusion protein, we have demonstrated an approach that may prove effective for engineering not only FGF family members, but other HP-binding molecules as well.

Keywords: fibroblast growth factor, heparan sulfate, heparin, proteoglycan, fusion protein

The heparin (HP)-binding, fibroblast growth factor (FGF) family consists of >20 members that play essential roles in regulating cellular differentiation, growth, and development[1]. Because FGF signal transduction requires formation of three-member complexes made up of FGF, heparan sulfate (HS)/HP, and FGF receptors (FGFR)[1], HS/HP-bearing proteoglycans (PGs) located on cell surfaces or in the extracellular matrix are important regulators of FGF biological activity. Indeed several FGF isoforms, including FGF-1, are known to require HP/HS in order to manifest their full mitogenic activity in vitro[2,3]. Not only does HP facilitate formation of biologically active FGF-1 dimers[4] and high-affinity binding of FGF-1 to FGFR[5], it also stabilizes the structure of FGF-1[6] and protects it from proteolytic inactivation[7]. Consequently, inclusion of free HP in culture medium is standard for in vitro application of FGF-1 (ref. 2). For broader use of FGF-1, however, FGF analogs that are less dependent on free heparin and are more resistant to inactivation would be desirable. Here, we describe such an FGF analog.

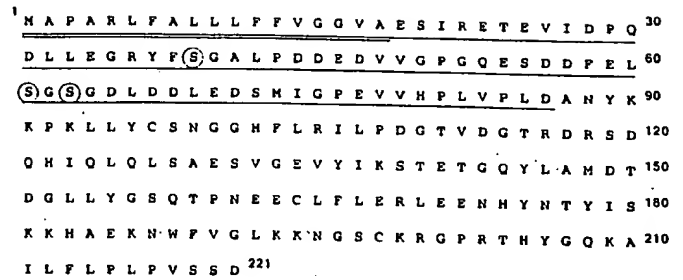
Results and discussion

Expression and characterization of PG–FGF-1 protein. To link HS sugar chains with FGF-1, the segment making up the signal and glycosaminoglycan (GAG) modification sequence within the core protein of syndecan-4 HS-PG[8] was fused with FGF-1 (Fig. 1A). The cDNA for the fusion protein, designated PG–FGF-1, was then subcloned into a eukaryotic expression vector, pMEXneo, and two lines of Chinese hamster ovary (CHO) cells, wild-type CHO-K1 and xylosyltransferase-deficient pgsA-745[9], were stably transfected. Medium conditioned by the transfectants was then analyzed for secreted PG–FGF-1 proteins by immunoblotting with anti-FGF-1 monoclonal antibody (mAb1)[10]. CHO-K1-conditioned medium contained proteins with FGF-1 antigenicity and molecular masses of 50–120 kDa (Fig. 1B, lane 1), whereas pgsA-745-conditioned medium contained FGF-1 antigenic proteins with molecular masses of about 29 kDa (designated mutPG–FGF-1; lane 5), which is the expected value for the secreted PG–FGF-1 core protein.

The PG–FGF-1 proteins were analyzed for their GAG composition. Digestion of PG–FGF-1 (Fig. 1B, lane 1) with a mixture of heparitinase I (HSase) and heparinase (HPase) yielded a strong protein band at 30 kDa, whereas another group of proteins remained at 50–80 kDa (lane 3). Digestion with chondroitinase ABC (CSase) yielded a protein band at 30 kDa, although most of the proteins remained at 50–80 kDa (lane 2). Digestion with a combination of HSase, HPase, and CSase yielded a protein band at 30 kDa (lane 4), which corresponds to that of mutPG–FGF-1 with basal GAG tetrasaccharides. On the other hand, the molecular mass of the mutPG–FGF-1 was unaffected by digestion with the enzyme mixture (lane 6). These results indicate that GAG glycosylation was the principal modification of PG–FGF-1 and that many, but not all, PG–FGF-1 proteins were glycosylated solely by HS; some samples were glycosylated solely by chondroitin sulfate (CS). That PG–FGF-1 is modified by both HS and CS is in good agreement with an earlier analysis of syndecan-4 sugar chains[11].

Fractionation of the PG–FGF-1 proteins. We next attempted to separate PG–FGF-1 proteins according to their affinity for HP. All of the PG–FGF-1 proteins were efficiently bound to a DEAE–Sepharose column in 150 mM NaCl at pH 7.4, confirming that all are highly modified with acidic groups (Fig. 2A). The DEAE-bound proteins were then eluted at 0.5 M NaCl and further fractionated on a heparin

A



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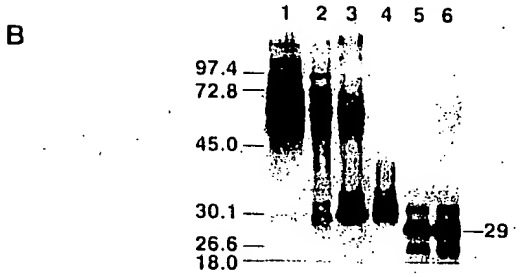

Figure 1. Structure and characterization of PG–FGF-1. (A) Primary structure of PG–FGF-1 core protein. Underlined amino acids make up a segment of human syndecan-4 ectodomain. Amino acids not underlined constitute the minimum active form of FGF-1. The circled serine residues are potential GAG attachment sites. The double underline indicates the predicted signal peptide for secretion. (B) Analysis of GAG modification. PG–FGF-1, and mutPG–FGF-1 proteins in culture medium from CHO-K1 or pgsA-745 transfectants were resolved by SDS–PAGE and immunoblotted with anti-FGF-1 (mAb1). Some samples were subjected to endoglycosidase digestion preceding analysis. PG–FGF-1 (lanes 1–4) and mutPG–FGF-1 (lanes 5 and 6) were either left untreated (lane 1, 1.2 ng; and lane 5, 0.4 ng) or digested with CSase (lane 2, 0.4 ng), a mixture of HPase and HSase (lane 3, 0.4 ng), or a mixture of all enzymes (lanes 4 and 6; 0.4 ng). Positions of molecular mass standards are indicated in kilodaltons. Three separate experiments yielded essentially the same results.

(HP)–Sepharose affinity column (Fig. 2B). Proteins that bound to HP–Sepharose were eluted at 1.2 M NaCl, pooled (fraction B), and analyzed by immunoblotting using mAb1. Proteins that bound to DEAE–Sepharose but not to HP–Sepharose were concentrated on a DEAE–Sepharose column, pooled (fraction U; Fig. 2C), and analyzed similarly. Fractions B and U both contained proteins with FGF-1 antigenicity and molecular masses of 50–120 kDa (Fig. 2D, lanes 1 and 5, respectively). Treatment of the fraction B (Fig. 2D, lane 1) with a mixture of HSase and HPase produced major bands at 30 kDa and 60 kDa (lane 3). Percentages of the modified FGF forms were calculated from their respective signal intensities and were normalized to the signal intensity of the 30 kDa band in lane 4, which contained no sulfated GAGs and was set to 100%. This calculation indicated that about 50% of the proteins in fraction B were glycosylated solely by HS. Treatment of the same proteins with CSase also produced major bands at 30 kDa and 60 kDa (lane 2); these results indicated that about 10% of the proteins were glycosylated solely by CS. Treatment with a mixture of all these enzymes produced an intense protein band at 30 kDa (lane 4), confirming that the major components of the modified PG–FGF-1 core protein were indeed HS and CS.

Similar analysis of the PG–FGF-1 proteins in fraction U (Fig. 2D, lane 5) showed that 80% were glycosylated solely by HS (lane 7), with only about 0.5% modified solely by CS (lane 6). Again, treatment with a mixture of all the enzymes produced an intense protein band at 30 kDa (lane 8). Thus, whereas PG–FGF-1 highly modified with HS is largely obtained as a HP–Sepharose-unbound fraction, the HP-bound fraction of PG–FGF-1 also harbors a considerable amount of HS.

Mitogenic activity of the HS-rich PG–FGF-1 toward Ba/F3 cells expressing FGFR in the absence of HP. To assess mitogenic

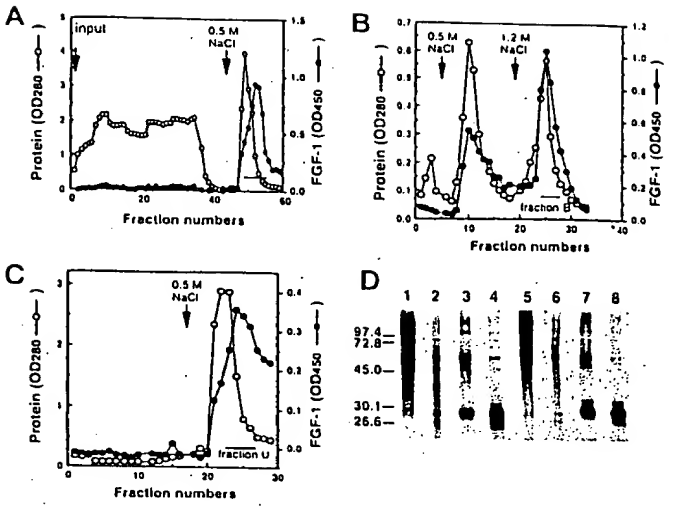

Figure 2. Separation of PG–FGF-1 subpopulations differing in their affinity for HP. PG–FGF-1 proteins secreted from CHO-K1 transfectants were first absorbed on a DEAE–Sepharose column. The DEAE-bound proteins were further fractionated by HP–Sepharose affinity chromatography (OD 280 nm; ○) and analyzed by ELISA (OD 450 nm; ●) and immunoblot. (A) DEAE–Sepharose chromatography. The column was loaded with secreted PG–FGF-1. Bound proteins were eluted by 0.5 M NaCl and collected (indicated by the bar). (B) HP–Sepharose chromatography. An aliquot of the DEAE-bound PG–FGF-1 fractions was applied to a HP–Sepharose column. The flowthrough fractions were saved (not shown), and the column was washed with 0.5 M NaCl. Tightly bound proteins were eluted by 1.2 M NaCl, and the eluate fractions (indicated by the bar; designated fraction B) were collected. (C) DEAE–Sepharose chromatography. The flowthrough fractions from (B) were loaded onto a DEAE–Sepharose column and washed with 0.1 M NaCl. Fractions eluted by 0.5 M NaCl (indicated by bar; designated fraction U) were then collected. (D) Proteins in the HP-bound (fraction B of (B), lanes 1–4) and unbound (fraction U of (C), lanes 5–8) fractions were digested with selected combinations of glycosidases, resolved by SDS–PAGE, and subjected to immunoblot analysis with anti-FGF-1 monoclonal antibody (mAb1)[10]. Lanes 1 and 5 are untreated fractions (6.9 ng); other lanes are fractions (0.69 ng) treated with CSase (lanes 2 and 6), HPase and HSase (lanes 3 and 7), or mixtures of the three enzymes (lanes 4 and 8). Positions of molecular mass standards are indicated in kilodaltons. Three separate experiments yielded essentially the same results.

activity of the different PG–FGF-1 fractions, we next examined the capacity of isolated PG–FGF-1 proteins to promote DNA synthesis in Ba/F3 cell transfectants expressing FGFR (FR-Ba/F3 cells). These cells have been used previously to investigate HP involvement in FGF signal transduction and are known not to express endogenous HS[12]. Interestingly, we found that the PG–FGF-1 proteins in fraction U stimulated DNA synthesis in FR-Ba/F3 cells in the absence of HP, but that similar activity was not elicited by the proteins in fraction B (Fig. 3A). In addition, mutPG–FGF-1 and simple FGF-1[13] (in CHO-conditioned medium) also failed to exert mitogenic effect. In the presence of HP, by contrast, fractions U and B were both potently mitogenic and stimulated DNA synthesis to similar degrees (Fig. 3B), confirming that the inability of fraction B proteins to stimulate DNA synthesis in the absence of HP (Fig. 3A) was not due to inactivation of the FGF-1 protein core. Treatment of PG–FGF-1 fraction U with HSase/HPase diminished its exogenous HP-independent mitogenic activity (Fig. 3C), whereas CSase had no effect (Fig. 3C). When free HP was added to the assay system, all the samples exhibited similar activity, regardless of the enzyme treatment (Fig. 3D). It was thus confirmed that the HP-independent activity of fraction U was provided by its HS chains, indicating that HS chains can at least in part substitute for free HP in the formation of the FGF–FGFR–HS complex necessary for transduction of mitogenic signaling.

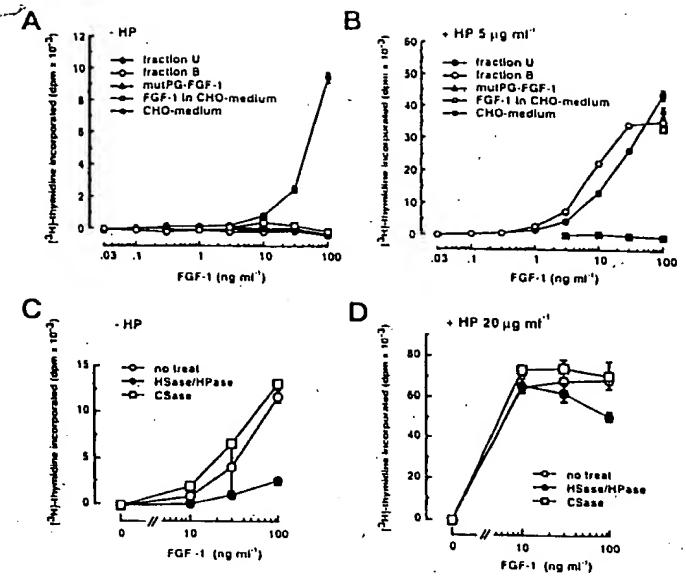

Figure 3. PG–FGF-1 Fraction U is mitogenic toward FR-Ba/F3 cells without addition of HP. (A and B) PG–FGF-1 protein fractions U and B, mutPG–FGF-1 protein, simple FGF-1 protein, and control samples were analyzed for their mitogenicity toward FR-Ba/F3 cells. [³H]Thymidine incorporation was assayed in the absence (A) and presence (5 μg ml⁻¹) (B) of free HP. Concentrations as FGF-1 domain are indicated on the abscissa. The control CHO-medium was tested at the same dilutions as FGF-1 in CHO-medium. (C and D), Fraction U was then treated with HSase/HPase or with CSase for 48 h at 4°C and analyzed for mitogenicity in the absence (C) or presence (D) of free HP (20 μg ml⁻¹). The concentrations as FGF-1 domain are indicated as the ones before enzyme treatment. Values represent means ± s.e. of triplicate samples. Similar results were obtained in three separate experiments.

The fact that the mitogenic activity was higher in the presence of free HP (Fig. 3B) than in the absence of free HP (Fig. 3A) indicates that the covalently bound HS sugar chains are imperfect substitutes for free HP. In the same vein, the ectodomain of syndecan-1 potentiates FGF-2 activity only after its degradation by HS-cleaving enzymes found in wound fluid[14]. When bound to the syndecan core protein, HP-like domains of HS sugar chains appear to have limited access to the HP-binding domains of FGF and/or FGFR. Alternatively, HS sugar chains in PG–FGF-1 may not be as appropriately modified with the sulfated groups that are very important to binding to FGF-1 as HP. It was of particular interest to us that although both PG–FGF-1 fractions U and B contain HS sugar chains (Fig. 2D, lanes 3 and 7), they differ substantially in their affinity for immobilized HP (Fig. 2B) and in their mitogenicity (Fig. 3). This suggests that the specific sites and/or structure of the HS modification affects the efficacy with which intramolecular HS substitutes for free HP.

Superior resistance of PG–FGF-1 to tryptic digestion. To examine whether conjugation with PG protects FGF-1 from proteolytic degradation, PG–FGF-1 was treated with trypsin. We compared PG–FGF-1 with simple FGF-1 also expressed by CHO cells and secreted into the conditioned medium[15]. As shown in Fig. 4, in contrast to CHO-expressed simple FGF-1, the majority of PG–FGF-1 proteins remained intact after exposure to trypsin. Moreover, treatment of the trypsinized samples with a mixture of HPase/HSase/CSase showed that the core proteins had remained largely intact or nearly so.

Potentiation of PG–FGF-1 mitogenic activity toward human endothelial cells by physiological degradation. As it was reported recently that physiological degradation converted the soluble syndecan-1 ectodomain from an inhibitor to a potent activator of FGF-2[14], we investigated whether degradation affects mitogenic activity of PG–FGF-1 using human umbilical vein endothelial cells (HUVEC),

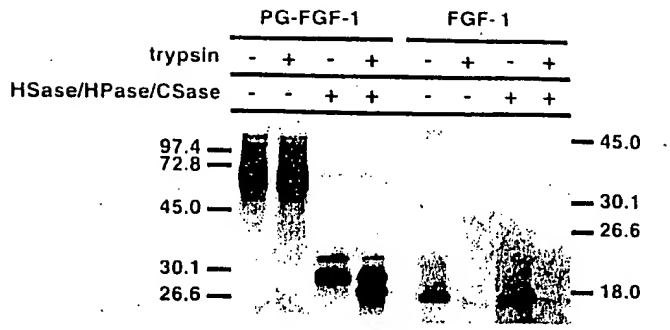

Figure 4. PG–FGF-1 is resistant to proteolytic digestion. PG–FGF-1 and FGF-1 treated with 0.0001% trypsin solution and untreated controls were digested with a mixture of HSase/HPase/CSase. The proteins were resolved by SDS–PAGE and immunoblotted with mAb1. Positions of molecular mass standards are indicated in kilodaltons. Three separate experiments yielded essentially the same results.

which are important physiological targets of FGFs. It was found that PG–FGF-1 dose-dependently stimulated DNA synthesis in HUVEC (Fig. 5A). Interestingly, the capacity of PG–FGF-1 to induce DNA synthesis in HUVEC was augmented by approximately threefold by treatment with a combination of trypsin and HSase, a treatment that mimics physiological degradation[14] (Fig. 5A, left panel, curves a and b). Treating CHO-expressed FGF-1 the same way, by contrast, abolished its biological activity (Fig. 5B, left panel). What is more, whereas PG–FGF-1 remained a mixture of molecules of 35–100 kDa, FGF-1 was mostly degraded (Fig. 5A, B, right panels). It was suggested that augmentation of PG–FGF-1 activity by degradation is likely due to the removal of poorly sulfated domains of HS and the recruitment of HP or effective HS sugar chain freed from the core protein by HSase in a manner analogous to the way HSase converted syndecan-1 from an inhibitor to an activator of FGF-2[14]. In addition, unlike simple FGF-1, the FGF-1 domain of PG–FGF-1 was protected from degradation by the presence of HS sugar chains. Thus, PG–FGF-1 is far superior to FGF-1 as a mitogen under physiologically degradative conditions (Fig. 5A, B) and it is suggested that PG–FGF-1 would be more suitable than FGF-1 for in vivo administration to sites of inflammation.

Whether HS sugar chain(s), covalently attached to FGF, can substitute for exogenous HS/HP has been a subject of interest. Intramolecular HS sugar chain exhibited many of the effects of exogenous HP: it enabled activation of FGFR by FGF-1, and protected FGF-1 from proteolytic degradation. In addition, PG–FGF-1 was superior to FGF-1 in some cases. Through construction of a biologically active proteoglycan-FGF-1 fusion protein, our study has shed light on a methodological approach that may prove effective for engineering other members of the FGF family as well as other HP-binding molecules. In addition, molecular modeling and/or cell engineering to precisely manipulate the FGF to HS sugar chain ratio and the structure of GAG should provide important new information about the stoichiometry and biological function of each member of the FGF–FGFR–HS complex mediating FGF signal transduction.

Experimental protocol

Cell culture. CHO-K1 cells were cultured in F-12 medium supplemented with 5% fetal bovine serum (FBS). HUVEC were isolated and cultured as described[3,16] in collagen type I-coated plastic dishes. PgsA-745 cells[9] were a kind gift from Dr. Jeffery D. Esko.

Transfection of Ba/F3 cells. FGFR-1c plasmid was a kind gift from Dr. David M. Ornitz. Ba/F3 cells were transfected with FGFR-1c plasmid by electroporation. Stable transfectants were established (FR-Ba/F3 cells), and the cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 10 ng ml⁻¹ FGF-1, and 10 μg/ml HP.

Construction of an expression vector for PG–FGF-1. An *Aor*51HI site was generated at the innermost end of the 5′ terminus of human FGF-1 cDNA by PCR using oligonucleotides No. 967 (5′-gcg tcg aca gcg cta att aca

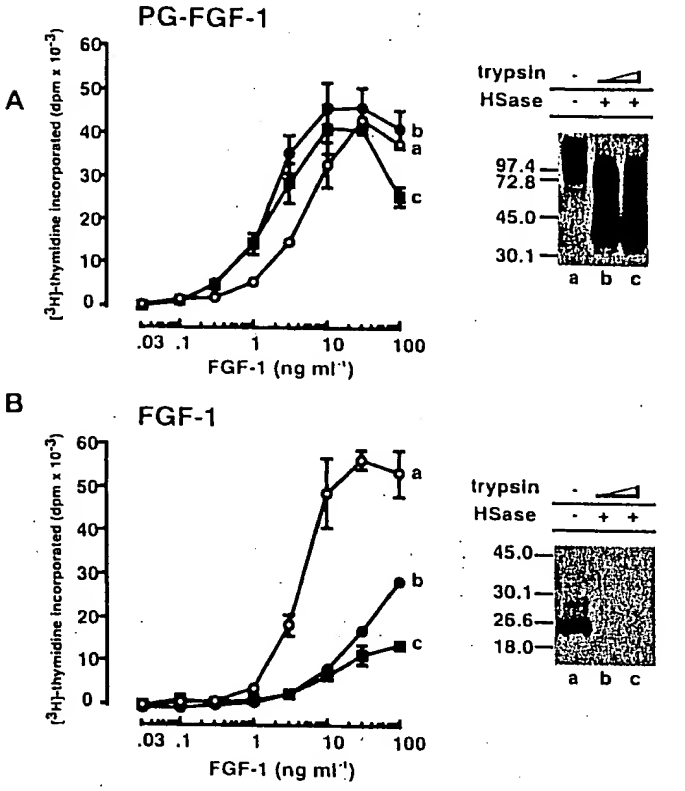

Figure 5. Mitogenicity of PG–FGF-1 toward HUVEC is potentiated by physiological degradation. Effects of physiological degradation on PG–FGF-1 (A) and FGF-1 (B). Left panels, Mitogenicity in HUVEC. Aliquots of PG–FGF-1 or CHO-expressed FGF-1 (200 ng ml^{-1}) were treated with buffer alone (○, curve a) or with HSase plus 0.0001% (●, curve b) or 0.001% trypsin (■, curve c). Mitogenic activity was then assayed in HUVEC in the presence of HP (20 μg ml^{-1}). The concentrations as FGF-1 domain are indicated as the ones before enzyme treatment. Similar results were obtained in three separate experiments. Right panels, immunoblotting. The enzyme-treated samples in the left panels were resolved by SDS–PAGE and immunoblotted with mAb1. Positions of molecular mass standards are indicated in kilodaltons. Three separate experiments yielded essentially the same results.

aga agc cca aac tc-3') and No. 630 (5'-cag aat tcg aat tct tta atc aga aga gac tgg-3') as primers; this also generated *Sal*I and *Eco*RI sites at the 5' and 3' termini. The amplified fragment was digested with *Sal*I and *Eco*RI and cloned into a pBluescript II KS(+) vector. A syndecan-4 cDNA plasmid was a kind gift from Dr. Tetsuhito Kojima. A partial cDNA for syndecan-4 was amplified by PCR using human syndecan-4 cDNA clone phR7A8[8] as a template and oligonucleotides No. 109 (5'-ttg tcg acc cac cat ggc ccc cgc ccg tct-3') and No. 111 (5'-ttg ata tct aga ggc acc aag gga tg-3') as primers, which generated *Sal*I and *Eco*RV sites at its 5' and 3' termini. The FGF-1 pBluescript plasmid was digested with *Sal*I and *Aor*51HI and ligated with the syndecan-4 cDNA fragment digested with *Sal*I and *Eco*RV. The nucleotide sequence of the clone (R/F cl. 25) was verified, and the PG–FGF-1 cDNA was excised by digestion by *Sal*I and *Eco*RI and subcloned into a pMEXneo expression vector[13].

Expression and fractionation of PG–FGF-1 proteins. CHO-K1 and pgsA-745 transfectants stably expressing PG–FGF-1 proteins were grown to subconfluence, the F-12/FBS was exchanged for ASF104 serum-free medium (Ajinomoto Co., Tokyo), which was then collected and renewed daily. Medium conditioned by the CHO transfectants was applied to a DEAE–Sepharose column (Amersham Pharmacia Biotech., Arlington Heights, IL), which was then washed extensively with PBS (pH 7.4) containing 0.001% 3-[(3-Chlolamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS). The bound proteins were eluted with Tris-CHAPS (10 mM Tris-HCl (pH 7.4) and 0.001% CHAPS) containing 0.5 M NaCl, and the eluate was diluted to 0.1 M NaCl and applied to HP–Sepharose column (Amersham Pharmacia Biotech.). The bound proteins were eluted with 1.2 M NaCl in Tris-CHAPS after washing with 0.5 M NaCl in Tris-CHAPS. The flowthrough proteins that did not bind to the HP–Sepharose column at 0.1 M NaCl were applied to the DEAE–Sepharose column and the bound proteins were eluted as described above. Selected protein-containing fractions were pooled and subjected to additional analysis. Concentrations of FGF-1, PG–FGF-1 and mutPG–FGF-1 were determined by ELISA using a polyclonal antibody against FGF-1 (R&D Systems, Minneapolis, MN). It was confirmed that this system resolved concentrations of GAG-modified and unmodified forms of PG–FGF-1 (data not shown). For western blots, samples were denatured with 2-mercaptoethanol and SDS, and resolved by SDS–10% polyacrylamide gel electrophoresis (SDS–PAGE). Separated proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH), blocked with 5% skim milk, and probed with mAb1. The signals were detected using peroxidase-conjugated anti-mouse IgG (Zymed Laboratories, South San Francisco, CA) and enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech.).

Endoglycosidase digestion. Samples were digested with endoglycosidases (Seikagaku Corp., Tokyo) using HSase (1 mU) or HPase (1 mU) in 0.1 M phosphate buffer (pH 7.0) or CSase (50 mU) in 0.1 M Tris-HCl buffer (pH 8.0) for 18 h at 37 °C unless otherwise noted.

Mitogenic activity assay. The [methyl-^{3}H]thymidine incorporation assay using HUVEC and FR-Ba/F3 was performed as described[12,16], except for use of 48-well plates.

Effects of physiological degradation on PG–FGF-1 mitogenicity. PG–FGF-1 and CHO-expressed FGF-1 (200 ng ml^{-1}) in PBS were incubated with trypsin (at the indicated concentrations) for 1 h at 37°C, after which the reactions were stopped with 1% FBS. Trypsinized samples were further incubated with HSase for 30 min at 37°C and transferred to 4°C, while control samples were incubated with buffer alone. Mitogenic activity of the samples toward HUVEC was measured in the presence of HP (20 μg/ml).

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